Genetic and epigenetic profile of sporadic pheochromocytomas

A Cascon, S Ruiz-Llorente, M F Fraqa, R Leton, D Telleria, J Sastre, J Jose Diez, G Martinez Diaz-Guerra, J A Diaz Perez, J Benitez, M Esteller, M Robledo

**Key points**

- Pheochromocytoma is a rare, neuroendocrine, chromaffin staining tumour that usually causes secondary hypertension by oversecretion of catecholamines.
- Recent studies have shown that 25% of patients with apparently sporadic tumours had germline mutations in one of the genes related to the disease (SDHB, SDHD, VHL, and RET).
- In this study we looked for somatic mutations in these pheochromocytoma-related genes in tumours to decipher their role in the pathogenesis of sporadic tumours. We found that 17% of tumours had mutations in the genes studied. All variants were also detected in the normal patient’s tissue, with no somatic changes with the exception of one RET substitution. When we compared the molecular data obtained through this study with clinical features, no correlation was found.
- Given that our results confirm the absence of somatic mutations affecting these genes, we developed a CpG island methylation analysis of the SDHB, SDHD, and VHL genes in order to find another inactivation mechanism affecting these pheochromocytoma-related genes.
- The study revealed an unmethylated promoter CpG island in all cases, suggesting that aside from a necessary search for other mechanisms involved in the possible somatic inactivation of these genes (gross deletions), other genes involved in the sporadic pathogenesis of this type of tumour must also exist.

**Materials and methods**

**Tumoral and normal tissue**

Tumoral samples from 35 patients with pheochromocytoma were collected anonymously with no information surrounding the existence of familial antecedents of the disease. This clinical information was subsequently obtained, along with normal tissue from the cases with variants. High molecular weight DNA was extracted from fresh frozen samples following standard procedures and also from paraffin embedded tumours using either the DNA Easy Tissue kit (Qiagen, Chatsworth, CA, USA) or a modification of a previously described protocol. Normal tissue specimens used in the methylation study were collected from autopsy material.

**Amplification and sequencing analysis**

The analysis was carried out by genomic DNA amplification by PCR and direct sequencing of all eight exons of SDHB, all four exons of SDHD, all three exons of VHL, and exons 10, 11, and 16 of RET, using primers and PCR conditions as previously described. By using DNA from 150–200 unrelated and unaffected individuals as a control population for single strand conformation polymorphism (SSCP) analysis, the pathogenic or polymorphic character of variants with unknown significance was defined. PCR products displaying mobility shift were subsequently sequenced using an automatic sequencer (ABI PRISM3700; Perkin Elmer Applied Biosystems, Foster City, CA, USA).

**Methylation specific polymerase chain reaction**

The methylation status of the SDHB, SDHD, and VHL CpG islands was analysed using the methylation specific

**Abbreviations:** PGL, paraganglioma; MSP, methylation specific polymerase chain reaction; SSCP, single strand conformation polymorphism

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Pheochromocytoma is a neuroendocrine chromaffin staining tumour that usually causes secondary hypertension by oversecretion of catecholamines. Clinically malignant pheochromocytomas are uncommon, although these tumours can metastasise by lymphatic or haematogenous pathways implied in the liver, lymph node, lung, and bone. Ten per cent of pheochromocytomas have been traditionally considered as hereditary tumours and may be associated with von Hippel-Lindau disease, multiple endocrine neoplasia type 2, or neurofibromatosis type 1. Recently, the presence of mutations in three (SDHB, SDHC, and SDHD) of the four genes comprising mitochondrial complex II has been associated with the development of the familial forms of these neuroendocrine tumours, either pheochromocytoma or paraganglioma. In fact, the distinction between sporadic and familial cases of pheochromocytoma has undergone a great change in recent months since it was reported that almost a quarter of patients with apparently sporadic pheochromocytomas may be carriers of germline mutations of these genes. The authors reported 24% of patients with germline mutations in VHL, RET, SDHD, or SDHB genes, thus challenging the traditional train of thought that proposed that only a minority of sporadic cases with mutations in the genes was involved in familial forms of the disease. Moreover, further study also describes the importance of germline mutations in patients with apparently sporadic parasympathetic paraganglioma (PGL).

In this study we have searched for somatic mutations in SDHB, SDHD, VHL, and RET in sporadic tumours to investigate the role of these genes in the pathogenesis of sporadic pheochromocytomas. In order to find additional mechanisms involved in the inactivation of these genes, we also performed a study of the methylation status of promoter CpG islands of VHL, SDHB, and SDHD to discover whether this inactivation mechanism affects pheochromocytomas.
polymerase chain reaction (MSP) technique. This assay distinguishes between unmethylated and methylated alleles in a given gene on the basis of sequence changes induced by sodium bisulphite treatment of DNA, which converts all unmethylated, but not methylated cytosines to uracil. Subsequently, the DNA region of interest is amplified with primer pairs specific for methylated versus unmethylated DNA. Normal lymphocytes, and placental DNA that had methylated in vitro with SSSI bacterial methylase were used as negative and positive control for methylation, respectively. The MSP primers used are summarised in table 1. In selected samples, the DNA methylation status was confirmed by bisulphite genomic sequencing, as previously described, using the primers summarised in table 1.

RESULTS

We screened 35 pheochromocytomas for the presence of somatic mutations in \textit{VHL}, \textit{RET}, \textit{SDHB} and \textit{SDHD}, using amplification analysis followed by PCR products sequencing. All findings in primary tumours are summarised in table 2.

We identified three \textit{RET} variants in three tumours: p.Cys611Phe in exon 10, p.Cys634Tyr in exon 11, and p.Met918Thr in exon 16. The mutational analysis of normal patient tissue with mutations revealed that p.Cys611Phe and p.Cys634Tyr had a germline origin, while p.Met918Thr resulted from a somatic change. By verifying the clinical data of the p.Cys611Phe and p.Cys634Tyr patients we found antecedents compatible with MEN2A. The first variation, p.Cys611Phe (table 2), was detected in a tumour sample of a female patient who had been diagnosed at 58 years of age with pheochromocytoma and C cell hyperplasia. The p.Cys634Tyr change was present in the primary tumour of a male subject diagnosed at 35 years of age with pheochromocytoma and the presence of bilateral or multiple tumours. The presence of positive familial antecedents of pheochromocytoma and \textit{VHL} or malignancy.

DISCUSSION

The presence of positive familial antecedents of pheochromocytoma and the presence of bilateral or multiple tumours are the main parameters associated with \textit{RET}, \textit{VHL}, \textit{SDHB}, or \textit{SDHD} germline mutations. Recent studies, however, report that about 20% of sporadic cases without such clinical features are associated with these genes, so the screening of mutations should be considered essential in all patients independent of their family history or the presence of tumour multiplicity and bilaterality. On the other hand, little is known about which genes are involved in the development of sporadic tumours, therefore in this study we firstly intended

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of primer</th>
<th>Primer sequence (5’ – 3’)</th>
<th>Untreated DNA (5’ – 3’)</th>
<th>Annealing temperature</th>
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<tr>
<td>MSP</td>
<td>SDHB</td>
<td>U, TAAATGGGTATGTGTTGTTATGTG,</td>
<td>CCAATGGCGATGCGCGCTACTTCG</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M, TGGTGTCTGGTCTGATGTCG</td>
<td>AGCCCCAGTGAGCAGCAGACTGGG</td>
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<tr>
<td></td>
<td>SDHD</td>
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<td>CGACAGCTGTTGTTGCGAGCCGC</td>
<td>60°C</td>
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<tr>
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<td>GAGCCGGCATCTCTCGTCGGAG</td>
<td></td>
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<tr>
<td></td>
<td>VHL</td>
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<td>GGCGAGCTTCTGTGGCGAGC</td>
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<tr>
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<td></td>
<td>M, TGAGGCTTCTGTGGCTTCT</td>
<td>GGGCGGAGCTTCCAGGCG</td>
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<tr>
<td>Bisulphite</td>
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<td>GGAATTTAGGTTAGGTTAGGTTAGGTTA</td>
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<tr>
<td></td>
<td></td>
<td>GACCCCTATGTACCGCACGCCAGCG</td>
<td>GACCCCTATGTACCGCACGCCAGCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDHD 1st set</td>
<td>GAGGGAGGAGGAGGAGGAGGAGGAGG</td>
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<tr>
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Table 1: MSP and bisulphite sequencing primers for the \textit{SDHB}, \textit{SDHD}, and \textit{VHL} promoters.
Online mutation report

The p.Glu42Ala and p.Ala90Thr substitutions (region of the tyrosine kinase domain). This mutation, deletion, eliminates a serine residue of the conserved amino acids often mutated in MEN2A. The three mutations found in RET (95%) to MEN2B, and has also been reported in some sporadic pheochromocytomas.24 25

Our results support these findings, and also confirm the absence of somatic mutations in sporadic pheochromocytomas.24 25

One tumour (~2.8%) was found to have an SDHB mutation, and was also germline. The change, a 3 bp deletion, eliminates a serine residue of the conserved and functional 4Fe-4S ferredoxin iron–sulphur binding domain of SDHB.5 The p.Glu42Ala and p.Ala90Thr SDHD substitutions (~5.7%) were germline (not previously described) and they did not affect conserved amino acids of the protein. We had no available material to perform further functional experiments, but as they were not detected in controls we considered these variants to be pathogenic changes.

Aside from other substitutions (g.129586_7insCTCTTT, p.Gly12Ser, p.Ser68Ser, g.112759G→A) previously described as polymorphisms,13 15 20 we found a new variant, p.Ser163Pro. This substitution was found in 2.3% of the control population, so it could be a rare polymorphism.

We found 17% of tumours to have mutations in some of the genes. All the identified changes were also found in the corresponding normal tissue with the exception of one somatic RET mutation. We have therefore found 14% of tumours to have germline mutations in some of the genes studied. This result differs from a previous study8 where 24% of apparently sporadic pheochromocytomas with germline mutations were found in one of the genes related to the development of these neoplasias (VHL, RET, SDHB, SDHD), although our study was of both a genetic and epigenetic nature. In addition, no more than one somatic RET change in tumours was found, nor were there somatic mutations in either the complex II genes or the VHL gene. It has been previously reported that somatic RET or VHL mutations are rare in sporadic pheochromocytomas,24 25 and SDHD mutations have been described as rare in neuroendocrine tumours.24 25 Our results support these findings, and also confirm the absence of somatic SDHB mutations in sporadic pheochromocytomas.27

As one of the classical inactivation mechanisms of tumour suppressor genes is the hypermethylation of promoter CpG islands,28 we attempted to discover whether this mechanism was involved in gene silencing. To date, the methylation status of SDHB remains unknown, and this study suggests that hypermethylation of the upstream CpG island of this
gene is not a frequent event in sporadic pheochromocytomas. On the other hand, it has been reported that VHL promoter methylation is not a common event in pheochromocytomas, and in this report we confirm that epigenetic silencing of the VHL gene in sporadic pheochromocytomas is not a prominent mechanism, in spite of its relevance in renal cell carcinoma.

SDHD, which is believed to be maternally imprinted by means of its inheritance pattern in PGL families, was not methylated at its canonical promoter CpG island in our pheochromocytomas. We also confirmed this finding in three different types of normal adrenal tissue. It has been suggested that it could be an epigenetic modification specifically affecting the carotid body in PGL families, although this event has not yet been molecularly demonstrated. Our results suggest that if methylation related imprinting exists for this gene, it can occur in another region. Moreover, the absence of methylation of this gene in both pheochromocytomas and normal adrenal tissue could indicate that tissue specific methylation is not the underlying mechanism of this pattern of inheritance, and therefore a different molecular mechanism must account for this model.

As no relation was found between the genetic alterations and the clinical characteristics of our series of pheochromocytomas, and given that methylation was not involved in the development of these tumours, other genes may be involved in the pathogenesis of these types of tumours. Further analysis is required in order to identify new candidate genes responsible for tumour pathogenesis of sporadic pheochromocytomas.

Figure 1 Bisulphite genomic sequencing of SDHD, SDHB, and VHL promoters in several representative human primary pheochromocytomas (13, 20, and 21). The localisation of the CpG island obtained with the NEWCPGREPORT software (EMOSS; http://mammoth.bii.a-star.edu.sg/emboss/index.html) is represented with a horizontal bar. The vertical bars represent the distribution of the CpGs at the SDHD, SDHB, and VHL CpG islands and the vertical arrow indicates the transcriptional start point. Black dots indicate methylated CpGs and white dots unmethylated. The position of the sets of bisulphite sequencing primers used for each gene is represented by grey (for the first set) and white (for the second set) horizontal arrows respectively. The position of the MSP primers is represented by horizontal arrows.

Figure 2 Representative MSP analyses of SDHD, SDHB, and VHL promoters in human primary pheochromocytomas (11, 12, and 21) revealing promoter unmethylation. The presence of a PCR band under the “U” or “M” lane indicates unmethylated or methylated alleles, respectively. In vitro methylated DNA (IVD) and normal adrenal (NA) DNA were used as positive methylated and unmethylated controls, respectively.
pheochromocytomas and to estimate the hereditary suscept-
ibility to developing this neoplasia.

ACKNOWLEDGEMENTS

We are grateful for the provision of tissue samples from the Tissue
Bank Network founded by the Molecular Pathology Programme of
the Spanish National Cancer Centre, along with the collaboration of
the following hospitals: Universitario 12 de Octubre, Clínico San
Carlos, Ramón y Cajal, Fundación Jiménez Díaz, Severo Ochoa,
Móstoles (Madrid) and Virgen de la Salud (Toledo). This work was
partially supported by the Fondo de Investigaciones Sanitarias project
FIS 00/0118. A Cascon is a postdoctoral fellow of the Excmo.
Ayuntamiento de Madrid. S Ruiz-Llorente is a predoctoral fellow of
the Excmo. J A Diaz Perez, M F Fraga, M Esteller, J Sastre,
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Received 22 September 2003
Accepted 22 September 2003

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*J Med Genet* 2004 41: e30
doi: 10.1136/jmg.2003.012658

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